Organophosphorus Insecticide Degradation by Heat-Labile Substances in Soil

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The persistence of several insecticides in nonsterile, heat-sterilized, and gamma radiation-sterilized soils was determined by gas chromatographic analysis of extracts. All of the pesticides degraded fastest in nonsterile soils and several insecticides [malathion, dichlorvos, Ciodrin (α-methylbenzyl 3-hydroxycrotonate dimethyl phosphate), and mevinphos] decomposed much faster in irradiated soil than in autoclaved soil. The degradation rates for the more stable organophosphorus insecticides including parathion, dimethoate, and diazinon were similar in irradiated and autoclaved soils. A heat-labile, water-soluble substance that accelerated the degradation of malathion was extracted with 0.2*N* NaOH from several nonautoclaved and radiation-sterilized soils. This substance was destroyed by heating soil suspensions for 10 minutes at 90° C. but most of its activity was retained in soils held at 25° C. for two to three months after radiation sterilization.

Microbial degradation of pesticides is frequently determined by measuring the differences in rates of degradation between autoclaved and nonautoclaved soil. Failure to maintain sterility for a sufficient period of time and physical-chemical changes produced by autoclaving often affect quantitative results. These objections can be overcome with other means of sterilization and subsequent aseptic procedures.

Gamma irradiation provides a means of sterilizing soil (McLaren *et al.*, 1957, 1962) without altering its physical and chemical properties as severely as autoclaving does (Eno and Popenoe, 1964). With gamma irradiation, soil temperatures remain within a few degrees of ambient during the sterilization procedure, thus making it possible to determine the effects of heat-labile substances upon the degradation of pesticides. This paper presents evidence for the presence of heat-labile, nonviable substances in soil which accelerate the breakdown of some organophosphorus insecticides.

METHODS AND RESULTS

Sultan silt loam, Chehalis clay loam, and an organic soil obtained from cultivated fields of western Washington were used in the study. Their preparation and properties have been described (Getzin and Rosefield, 1966). Soils were sterilized with gamma radiation, heat, or a combination of the two methods. The radiation source was provided by a Mark II cobalt-60 unit located in the Department of Fisheries, University of Washington, Seattle (Liston *et al.*, 1963). Soil samples received 4-mrad doses of gamma radiation at a rate of 250,000 rads per hour. A small amount of sterilized soil from each sample was distributed on potato-dextrose or soil-extract agar plates immediately prior to insecticide treatment and at the time of analysis. Culture plates were examined for contamination several days later. The 4-mrad dose of radiation sterilized all soils but occasionally a sample became contaminated during application of the insecticide. Data from contaminated samples were discarded, although differences in rates of insecticide breakdown between sterile and nonsterile replicates were not significant.

For heat sterilization, moist soil samples were autoclaved 1 hour at 15 p.s.i. three times at weekly intervals. After initial experiments, one 20-minute autoclaving treatment of sterile irradiated soil replaced the severe heat-sterilization procedure for destroying heat-labile substances which accelerated the breakdown of some insecticides. Results obtained from the two methods were similar.

Insecticide emulsions prepared from analytical or technical grade toxicants were applied aseptically. For most experiments 4-ounce prescription bottles containing 10 cc. of moist soil (equivalent to 12 and 10 grams of air-dried Sultan silt loam and Chehalis clay loam, respectively) were irradiated, autoclaved, or kept as nonsterile controls. The insecticides were applied with disposable syringes at rates of 15 to 25 μg. per cc. of soil with sufficient amounts of H_2O to maintain soil samples at their moisture equivalent as determined by the method of Briggs and McLane (1907). The bottles were capped and agitated to distribute the insecticide emulsion throughout the soil. Samples were aged at 25° C. for the desired times (Tables I and II) before extracting the pesticide residues. All treatments included triplicate samples.

Samples were extracted directly in the 4-ounce prescription bottles after removing a small amount of soil for the sterility assay on potato-dextrose agar. Approximately 25 grams of anhydrous Na_2SO_4 and 25 ml. of purified acetone were added to each container. The bottles were shaken for 15 minutes and 50 ml. of purified hexane (Skellysolve B) were added. The bottles were again shaken for 45 minutes and centrifuged. Ten milliliters of extract were removed and saved for quantitative analysis.

The insecticide residues were measured by gas-liquid chromatography with a phosphorus detector attached to a

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Varian Aerograph 600-D gas chromatograph containing a 5-foot × 1/8-inch borosilicate glass column packed with QF-1 on 60- to 80-mesh Gas Chrom Q. Operating parameters were dependent upon the insecticide and the sensitivity of the detector. Helium was used as a carrier gas at a flow rate of 18 ml. per minute and the hydrogen flow varied from 20 to 25 ml. per minute. The injector column temperature was set at 230° to 250° C. and oven temperatures were adjusted between 155° and 240° C. to obtain insecticide retention periods within 2 to 4 minutes. With an electrometer setting of 1.6×10^{-10} afs, 1 to 20 ng. of insecticide were required to produce 25% full scale deflection of a 1-mv. Leeds & Northrup Model W recorder. Soil extracts were usually evaporated to dryness and diluted accordingly with acetone to stay within the linear range of the detector. Recovery of insecticides from fortified, autoclaved soil ranged from 80 to 95%. Only 60 to 70% recovery of unstable insecticides was obtained from nonsterile soil extracted immediately after treatment.

Effect of Gamma Radiation upon Insecticide Stability. It would be convenient and desirable to irradiate the soils after applying the insecticides to assure sterility and to obtain better distribution of the insecticides by applying materials with a sprayer rather than a syringe or pipet. Therefore, the effect of irradiation upon the stability of malathion, diazinon, and Zinophos (*O,O*-diethyl *O*-2-pyrazinyl phosphorothioate) was determined.

Insecticide emulsions were sprayed onto 200-cc. volumes of Sultan silt loam (240 grams) and acid-washed quartz sand (340 grams) in an open-faced rotary blender. Equivalent 10-cc. samples of the insecticide-treated materials were weighed into each of 18 4-ounce prescription bottles. One half the bottles were evacuated and refilled with helium to reduce the quantity of O_2 present. It was suspected that ozone formed during irradiation might contribute to insecticide decomposition. Triplicate samples of each insecticide in soil and in sand under normal and helium atmospheres were irradiated with 1 mrad, and immediately extracted to remove the pesticide residues for analysis.

The insecticides were partially decomposed by the radiation treatment. Decomposition above the nonirradiated controls for diazinon, malathion, and Zinophos averaged 15, 27, and 42%, respectively, in quartz sand. In soil, decomposition levels for diazinon and Zinophos were 17 and 24%, respectively. Rapid degradation of malathion in nonirradiated soil controls prevented estimation of its breakdown by radiation. Decomposition of insecticides in a helium atmosphere was only slightly less than in the normal atmosphere, suggesting that degradation is not entirely due to the effect of ozone. Because the three insecticides were partially decomposed by gamma irradiation at rates insufficient to sterilize soils, all further attempts to treat soils with insecticides prior to irradiation were abandoned.

Persistence of Insecticides in Irradiated and Autoclaved Sultan Silt Loam. The persistence of malathion, Zinophos, and diazinon was compared in nonsterile, irradiated, autoclaved, and autoclaved irradiated soil. The insecticides were applied 24 hours after completion of the radiation treatment. Malathion residues were extracted 1 week later, whereas Zinophos- and diazinon-treated samples were incubated for 4 weeks. The irradiated and autoclaved samples remained sterile throughout the incubation period.

Comparisons of insecticide persistence in autoclaved and irradiated soils (Table I) provide evidence for the existence of one or more heat-labile substances capable of accelerating the decomposition of malathion and Zinophos. Most of the malathion decomposed in nonsterile and irradiated soils after 1 week but persisted in autoclaved soils. Zinophos also degraded more rapidly in irradiated soils than in autoclaved soils. No significant difference in the degradation of diazinon between treatments was observed. Residues of the compounds in the autoclaved soil and the irradiated autoclaved soil were similar, indicating that insecticide persistence was not affected by chemical changes in the soil which may have resulted from radiation.

Decomposition of Insecticides in Nonsterile, Irradiated, and Autoclaved Chehalis Clay Loam. The persistence of several insecticides was compared in nonsterile, irradiated, and autoclaved irradiated Chehalis clay loam. Chehalis clay loam was selected because many organophosphate insecticides degrade faster in it than in other soils commonly used in this laboratory. The insecticides used were either short-lived in soil or known to be more persistent in autoclaved soil than in nonautoclaved soil. Irradiated soil samples were incubated for 2 weeks before applying the insecticides. The stability of the insecticides in nonsterile soil determined the incubation interval between treatment and analysis. The least stable compounds, malathion, Ciodrin (α-methylbenzyl 3-hydroxycrotonate dimethyl phosphate), dichlorvos, and mevinphos were analyzed 24 hours after soil treatment; methyl parathion and GS 13005 {*S*-[(2-methoxy-5-oxo-Δ-1,3,4-thiadiazolin-4-yl)-methyl]*O,O*-dimethyl phosphorodithioate} were incubated for 1 week, parathion and dimethoate for 2 weeks, and Zinophos and Dursban [*O,O*-diethyl *O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate] were analyzed 4 weeks after treatment.

All of the insecticides degraded more rapidly in nonsterile soil than in irradiated soil (Table II), which suggests that microorganisms were partly responsible for degradation of the compounds. The difference between the amount of each insecticide decomposed in irradiated soil and in autoclaved soil is attributed to nonviable, heat-labile substances. Heat-labile factors were primarily responsible for the loss of malathion and dichlorvos in soil. These substances also caused significant losses of

Table I. Degradation of Insecticides in Irradiated, Autoclaved, and Nonsterile Sultan Silt Loam

Soil Treatment	% Insecticide Degraded[a]		
	Malathion	Zinophos	Diazinon
Nonsterile	99	68	45
Autoclaved	11	23	42
Irradiated	98	31	44
Irradiated and autoclaved	10	22	44
Standard deviation	5	3	3

[a] Malathion samples extracted after 1 week; diazinon and Zinophos analyzed after 4 weeks.

Ciodrin and mevinphos and smaller losses of methyl parathion, Zinophos, and Dursban. Dimethoate, parathion, and GS 13005 did not degrade faster in irradiated soil than in autoclaved soil.

Stability of Heat-Labile Factor in Soil. Samples of Sultan silt loam were irradiated and assayed periodically for the presence of the heat-labile factor which enhances the decomposition of malathion. Insecticide emulsions were applied aseptically to irradiated and autoclaved-irradiated soil samples 1, 7, 28, and 84 days after sterilization. Malathion residues were extracted 0 and 4 days after treatment.

The amount of malathion degraded after 4 days in soil treated 1, 7, 28, and 84 days after radiation sterilization was 98, 90, 83, and 84%, respectively. In autoclaved irradiated soil, malathion loss averaged 8% for the four analysis periods. Stability of the heat-labile factor which accelerates malathion degradation was established, since the insecticide breakdown in irradiated soil greatly exceeded that in autoclaved soil 84 days after sterilization.

Inactivation Temperature of Heat-Labile Substance in Soil. Preliminary trials revealed that assays for activity could be conducted conveniently with aqueous soil suspensions in place of the normal soil treatments and the former method was used to determine the inactivation temperature of the fraction which accelerated the decomposition of malathion.

One-gram air-dried equivalents of moist, radiation-sterilized Sultan silt loam were suspended in 4 ml. of H_2O buffered at pH 6.3 in 125 × 20 mm. screw-top culture tubes. Three tubes of the soil suspensions were each heated for 10 minutes in a water bath at one of eight temperatures (25°, 40°, 50°, 60°, 70°, 80°, 90°, and 100° C.) and then immersed in ice water until the suspensions reached room temperature. The series also included a set of samples autoclaved at 15 p.s.i. for 20 minutes and a buffered control without soil. Forty micrograms of malathion in 1 ml. of H_2O was added to each tube. The tubes were shaken at room temperature for 24 hours and the malathion residues were extracted with 10 ml. of hexane and measured by gas chromatography.

The amount of insecticide decomposed in soil suspensions subjected to the various temperatures is an indicator of the amount of heat-labile activity present. The percentage of malathion degraded after 24 hours in suspensions previously incubated for 10 minutes at 25°, 40°, 50°, 60°, 70°, 80°, 90°, and 100° C. was 90, 89, 87, 80, 79, 69, 12, and 8%, respectively. In autoclaved suspensions and control solutions without soil, 6% of the insecticide decomposed after 24 hours. Increased deactivation of the factor occurred with increasing temperatures. The major destruction of the activity occurred between 70° and 90° C. in soil suspensions.

Extraction of Heat-Labile Factor from Soil. To extract a heat-labile fraction which accelerated the decomposition of malathion, 20 grams of air-dried equivalent of moist soil were shaken with 100 ml. of 0.2*N* NaOH in a 250-ml. centrifuge bottle for 1 hour. After removing the heavier soil materials by centrifugation at 1000 G, the dark-brown, alkali-soluble fraction was separated from the remaining colloidal residue by centrifugation at 17,000 G for 10 minutes and immediately brought to pH 8.0 with 3*N* HCl. To assay for activity 4-ml. aliquots of solution (1 gram soil equivalent) were placed in centrifuge tubes with 1 ml. of tris buffer (pH 7.9) and 40 μg. of malathion. Each volume was brought to 10 ml. and the tubes were incubated at 25° for 20 hours. Malathion residues were extracted with 10 ml. of hexane for chromatographic analysis. The extracted soil residues were washed twice with water buffered at pH 7.9 and 1-gram soil equivalents were similarly assayed for activity.

The degradation of malathion was measured in nonsterile, irradiated, and autoclaved soils, alkali extracts, and extracted soil residues of Sultan silt loam, an organic soil, and Chehalis clay loam. Irradiated soils were incubated for 2 months at 25° before extraction with NaOH. The pH of all buffered suspensions and solutions was recorded before and after the 20-hour incubation period. The hydrolysis of malathion was also determined in buffered aqueous controls containing no soil or alkaline extract.

Examination of the data in Table III indicates a substance was extracted from nonautoclaved soils with 0.2*N*

Table II. Degradation of Insecticides in Nonsterile, Sterile Irradiated, and Sterile Autoclaved Irradiated Chehalis Clay Loam

Insecticide	Incubation Period	% Insecticide Degraded: Autoclaved soil	Irradiated soil	Nonsterile soil	Standard Deviation
Malathion	1 day	7	90	97	4
Ciodrin	1 day	4	34	87	5
Dichlorvos	1 day	17	88	99	2
Mevinphos	1 day	1	38	95	3
Methyl parathion	1 week	20	26	95	4
GS 13005	1 week	17	20	50	4
Parathion	2 week	17	16	35	3
Dimethoate	2 week	18	20	77	3
Zinophos	4 week	17	24	71	5
Dursban	4 week	33	38	62	4

Table III. Degradation of Malathion after 20 Hours at pH 7.9 in Three Soils, Their 0.2*N* NaOH Extracts, and Extracted Soil Residues

Treatment	% Malathion Degraded: Chehalis clay loam	Organic soil	Sultan silt loam
Nonsterile soil	100	94	95
Irradiated soil	100	80	64
Autoclaved soil	25	22	29
Nonsterile NaOH extract	58	88	54
Irradiated NaOH extract	67	75	35
Autoclaved NaOH extract	25	25	24
Nonsterile extracted residue	48	75	40
Irradiated extracted residue	46	57	35
Standard deviation	4	5	3

NaOH which accelerated the degradation of malathion. The insecticide degraded faster in nonsterile and irradiated soils, and in alkali extracts of the soils, than in autoclaved soils and their extracts. After extraction with 0.2*N* NaOH the washed soil residues still retained some ability to degrade malathion. Efforts to improve the efficiency of the extraction procedure with higher concentrations of NaOH were not attempted.

The percentage of malathion hydrolyzed in buffered water controls (pH 7.9) after 20 hours at 25° C. averaged 25 ± 2%. This was similar to the degradation rate of the insecticide in the autoclaved soil and soil extracts, which indicates the heat-labile substance is the only nonviable factor that affects the stability of the pesticide. Boiling the soluble fractions from nonautoclaved soils completely destroyed the active component.

Microorganisms may have contributed to the greater loss of malathion in nonsterile soils than in irradiated soils. However, since nonsterile soil extracts degraded malathion faster than irradiated soil extracts, part of the differences probably were due to destruction of the active factor caused by the radiation treatment, or by aging. A previous trial indicated malathion is degraded slightly faster in newly irradiated soil than in aged irradiated soil.

The NaOH extracts were also assayed for activity at pH 7.9 with Ciodrin, dichlorvos, and mevinphos. None of these insecticides were degraded by the alkali extract which accelerated the breakdown of malathion.

DISCUSSION

Although several compounds degraded faster in irradiated soil than in autoclaved soil, a soluble heat-labile factor extracted with 0.2*N* NaOH only accelerated the decomposition of malathion. It served as the sole substrate in assaying solutions for activity while attempting to isolate the substance from soil. That other insecticides were not degraded by the NaOH extract suggests the presence of additional heat-labile active fractions which are either inactivated or not extracted by 0.2*N* NaOH.

The nature of the substance in soil which degrades malathion is under investigation. The substance was not extracted with 1*M* sodium pyrophosphate, oxalate, chloride, bicarbonate, and citrate, 0.1% EDTA, 0.1*N* aluminum sulfate, ion exchange resins, charcoal, or buffered solutions within a pH range of 2 to 10. These reagents and adsorbents did not inactivate the material because extracted soil residues retained the capacity to degrade malathion. The organic nature of the active fraction is suggested by the fact that neither pure clays nor soil treated with H_2O_2 exhibited the capacity to degrade the insecticide. Organic substances could be derived from decaying vegetation or microorganisms.

Enzymes are a possible explanation for the activity in irradiated soil. Even though radiation-sterilized microorganisms lose the ability to multiply, many of their enzymatic and biochemical activities continue for some time thereafter (McLaren *et al.*, 1957; Peterson, 1962). Furthermore, enzyme fractions which degrade malathion have been extracted from soil organisms (Matsumura and Boush, 1966). However, enzymes are usually inactivated by 0.2*N* NaOH. The substance that affects malathion retains its activity for a week or more when held in 0.2*N* NaOH at room temperature.

The question immediately arises: Is the active heat-labile fraction associated only with living organisms and radiation-sterilized organisms or, is it a permanent constituent of soil? The fraction which accelerates the breakdown of malathion is stable (85% of original activity remained after 3 months in sterile irradiated soil) which implies the substance exists as a permanent part of the soil complex. Further evidence of its stability is indicated by the retention of activity in soils or solutions following treatment with dilute acid (pH 2) or 0.2*N* NaOH.

Not all soils contain an alkali-extractable fraction capable of degrading malathion. To date, an active substance has been extracted from four of five soils collected from western Washington, but not from four soils obtained from the arid eastern region of the state.

The origin, location, and persistence of the heat-labile substances are important in quantitative assays of insecticide degradation in soil by microorganisms. If the substances which accelerate the breakdown of insecticides are associated only with living or radiation-sterilized organisms, soils should be sterilized with heat to determine the importance of microbial degradative activity. However, if the active heat-labile materials, regardless of their origin, exist outside of living organisms, it is necessary to utilize procedures other than heat to sterilize soil for microbial degradation studies.

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